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MULTIPLE NUCLEI DURING EARLY OOGENESIS IN *FLECTONOTUS PYGMAEUS* AND OTHER MARSUPIAL FROGS

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In the great majority of amphibians yet investigated the occurrence of oocytes with more than one nucleus or more than a single meiotic figure is exceptional (Humphries, 1956, 1966; Parmenter, Derezin and Parmenter, 1960; Humphrey, 1963). In the tailed frog *Ascaphus truei*, however, Macgregor and Kezer (1970) found that oogenesis regularly involves oocytes with eight nuclei, all but one of which disappear before the final stages of oogenesis. When a related species, *Leiopelma hochstetteri*, was investigated by Robinson, Stephenson and Stephenson (1973), only a single binucleate oocyte was found among 26 oocytes examined. In the present paper the occurrence of multinucleate stages as a regular feature of oogenesis in several genera of marsupial frogs of South America is reported. The multinucleate condition is associated with the early stages of oogenesis; in large, yolky oocytes only one nucleus is present.

Most of the marsupial frogs are inhabitants of the humid forests of South America. In these frogs the aquatic larval stages are reduced or eliminated altogether, a phenomenon associated with the fact that the female carries the embryos on her back, either within a pouch of integument or in shallow depressions of the skin. The genera *Flectonotus*, *Gastrotheca*, and *Amphignathodon* are characterized by pouches, while in *Fritziana, Cryptobatrachus, Stefania* and *Hemiphractus* the embryos are carried in depressions of the skin. For a list of these frogs with locality and references of taxonomic interest see the work of Duellman (1976).

Relatively little information is available regarding reproduction and development in marsupial frogs. The relationship between mother and embryos has been studied in some detail in *Gastrotheca riobambae*, a species that carries the embryos in the pouch up to the tadpole stage (see del Pino, Galarza, de Albuja, and Humphries, 1975, for references). Among the species of *Gastrotheca* that carry the embryos to the young froglet stage, *Gastrotheca ovifera* is the best known (see Mertens, 1957, for a description of its life history and references).

This report describes some features of oogenesis in 33 species of marsupial frogs that correspond to the described species of *Amphignathodon* and *Hemiphractus* as listed by Duellman (1976), to two (of three) species of *Flectonotus*, one (of six) species of *Stefania* and 20 (of 32) species of *Gastrotheca*. In addition, the ovaries of three unnamed species of *Gastrotheca* were also analyzed. The peculiarities of oogenesis have been studied in more detail in *Flectonotus pygmaeus, Gastrotheca ovifera* and *Gastrotheca* sp., an unnamed species from Venezuela, since in these instances both living and preserved specimens were available. Living specimens of *G. marsupiata, G. excubitor, G. mertensi* and *G. plumbea* were also available, but only in limited numbers. Study of other frogs was restricted to museum specimens, with the exception of *G. riobambae*, in which the ovary had been studied previously (del Pino and Sánchez, 1977).

Materials and Methods

Specimens examined and laboratory care of living frogs

Flectonotus pygmaeus, Gastrotheca ovifera and *Gastrotheca* sp. were collected at Estación Biológica de Rancho Grande, Maracay, Estado de Aragua, Venezuela in November 1975 and transported alive to the laboratory in Quito. In addition, several specimens preserved in 10% formalin or in Bouin's picro-formol were available for study. *Flectonotus pygmaeus* gives birth to advanced tadpoles, while *G. ovifera* and *Gastrotheca* sp. give birth to froglets. For this analysis the ovaries

Table I

Size of ovarian oocytes of Flectonotus pygmaeus *at various reproductive stages.*

Reproductive stage	Oocyte diameter (μm)	Number of large oocytes			Number of embryos in the pouch
		Left ovary	Right ovary	Total	
Juvenile					
Without pouch	500	4	7	11	
	500				
Open pouch	1200	0	5	5	
	1500				
	2000	3	3	6	
	2500	3	6	9	
Adult					
Closed pouch	3000	4	9	13	
	3000	6	5	11	
	3000	5	3	8	
Pregnant					
4 days	800	3	3	6	6
8 days	1000	6	7	13	8
18 days	500				6
20 days*	1500	3	2	5	7
20 days	2000	4	5	9	8
23 days	2000	6	6	12	11
28 days	500				7
At birth	2000	1	11	12	11
	2000	1	5	6	5
	2000	1	4	5	8
	2500	3	5	8	7
	2000	4	5	9	6
After birth					
2 days	1500	6	2	8	7
3 days	500				8
4 days	1500	2	3	5	6
Mean ± s.d.		3.5 ± 1.8	5.0 ± 2.3	8.5 ± 2.8	7.4 ± 1.7

* Frog was killed 20 days after ovulation; mating did not occur.

of 25 *F. pygmaeus* females (Table I) and those of five *G. ovifera* and two *Gastrotheca* sp. were studied.

Juvenile specimens of *F. pygmaeus* were kept in a humid terrarium at 20° C (range 17–21° C) and were fed *Drosophila* once or twice a day. The frogs lived for nearly a year. Reproductive activity occurred spontaneously after about five months of captivity. Three females became pregnant; a fourth deposited eggs but did not mate. In the latter case, the eggs were deposited on the wall of the terrarium. One of these eggs was fixed with Bouin's picro-formol for cytological examination. Under laboratory conditions, the period of incubation in the pouch lasts about 29 days; advanced tadpoles then emerge from the pouch and metamorphose after about 30 days. Incubation in the pouch of this species is considerably abbreviated in comparison with the situation in *G. riobambae,* where development in the pouch lasts nearly four months (del Pino *et al.*, 1975).

Most of the museum specimens that were examined belong to the Museum of Natural History of the University of Kansas (KU). Other specimens belong to The American Museum of Natural History (AMNH), Museo de la Estación Biológica de Rancho Grande (EBRG), the Field Museum of Natural History (FMNH), Museo de la Fundación Miguel Lillo (MFML), the Museum of Zoology of the Louisiana State University (LSUMZ) or the Museum of Natural History of the University of Southern California (USC). Information regarding the museum specimens is presented below.

AMPHIGNATHODON: *A. guentheri* KU-164228, Ecuador, Pichincha: 5 km ESE Chiriboga, 2010 m.

CRYPTOBATRACHUS: *C. fuhrmanni* KU-169378, Colombia, Norte de Santander, 32 km W Sardinata, 1050 m.

FLECTONOTUS: *F. fissilis* KU-92240, Brazil, Guanabara: Rio de Janeiro, Tijuca.

GASTROTHECA: *G. argenteovirens* KU-144123, Colombia, Cauca: Road to Pacific coast from El Tambo, 2170 m. *G. cavia* KU-148534, Ecuador, Imbabura: Laguna de Cuicocha, 2890 m. *G. ceratophrys* KU-77016, Panamá, Darien, Laguna, 820 m.; AMNH-90984. *G. christiani* MFML-02117, MFML-02117-5, Argentina, Jujuy, Abra de Cañas V. Grande. *G. cornuta* KU-169394, Colombia, Cauca: La Costa, El Tambo, 1000 m. *G. excubitor* KU-163135, KU-163140, Perú, Abra Acanacu, 25 km NNE Paucartambo, 3520 m. *G. gracilis* MFML-01972, Argentina, Tucumán, Road Tafí del Valle km 41. *G. griswoldi* KU-138221, Perú, Junin, Mayupampa, 21 km N La Oroya, 3400 m; KU-138227, Perú, Junin, Comas 3220 m. *G. lojana* KU-138234, Ecuador, Loja, 10 km W Loja 2500 m; KU-142608, Ecuador, Loja, 5.5 km W Loja, 2330 m. *G. marsupiata* KU-138399, Perú, Huancavelica: Huancavelica 3780 m; KU-139187, Perú, Cuzco, 14.5 km S Paucartambo, 3450 m. *G. mertensi* KU-140386, Colombia, Nariño: La Victoria, 2700 m. *G. microdisca* KU-154610, Brazil, São Paulo: 10 km NW Caraguatatuba 500–750 m. *G. monticola* KU-138402, Ecuador, Azuay: Girón 2240–2500 m; KU-142610, Eucador, Loja: Saraguro, 2510 m. *G. ochoai* KU-138668, Perú, Puno: Ollachea, 53 km W. Macusani, 2800 m. *G. ovifera* KU-125372, Venezuela, Distrito Federal de Caracas; KU-13338, Venezuela, Aragua: Quebrada, 0.5 km E. Res. Sta. at Rancho Grande, 1075 m. *G. peruana* KU-138444, Perú, Huanco 5 km NE La Union 3100 m; KU-138494, Perú, Cajamarca: Cajamarca, 2800 m; KU-138526, Perú, Ancash, Chavin de Huantar 3230 m. *G. plumbea* KU-132414, Ecuador, Cotopaxi: Pilaló 2460–2580 m; KU-164230, Ecuador, Pichincha: 9.5 km NW Nono 2530 m. *G. testudinea* KU-163276, Perú, Ayacucho: Tutumbaro, Rio Piene, 1840 m. *G. weinlandii* KU-146042, Ecuador, Morona Santiago: Rio Piúntza, 1830 m. *Gastrotheca* sp., FMNH-39889. *Gastrotheca* sp., LSUMZ-32049. *Gastrotheca* sp., EBRG-48240, Venezuela, Aragua: Res. Sta. at Rancho Grande.

HEMIPHRACTUS: *H. bubalus* KU-169426, Colombia, Putumayo: 10.3 km W El Pepino, 1440 m. *H. fasciatus* KU-93503, Panamá, Altos de Pacora; KU-116353, Panamá, San

Blas: Camp Summit, 300–400 m. *H. johnsoni* USC-716, Perú. *H. proboscideus* KU-123139, Ecuador, Napo: Santa Cecilia, 340 m. *H. scutatus* KU-147118, Ecuador, Morona Santiago: Rio Piúnza, 1400 m.

STEFANIA: *S. scalae* KU-167222, Venezuela, Bolivar: Paso de El Danto, El Dorado-Sta. Elena de Vairén road, km 117–119 (100–1150 m); KU-16239, KU-167248, Venezuela, Bolívar: El Dorado-Sta. Elena de Vairén road, km 112, 860 m.

Cytological procedures

The nuclei of ovarian oocytes were observed in both living and histological preparations. Oocytes from 100–500 μm are somewhat transparent and can be studied intact. Observations were made on such oocytes using a depression slide or a standard slide on which the coverglass was slightly elevated. Individual nuclei were observed similarly with phase contrast microscopy after rupturing the oocyte in Ringer's solution or a 5:1 mixture of 0.1 M NaCl and 0.1 M KCl.

Ovarian tissue fixed in 10% formalin or in Bouin's picro-formol, as well as that from museum material, was embedded and cut into sections of 10 μm thickness and stained with Harris' hematoxylin. Alcoholic eosin yellow was used as the counterstain. Some preparations were made using the standard Feulgen procedure.

The number of nuclei per oocyte was estimated by counts in the sectioned material. The section interval for the counts was decided by measurements of nuclear diameter. Since the size of nuclei within a single oocyte is highly variable at some stages, the estimates of total nuclear number are subject to considerable error. Sections of museum material were unsuitable for nuclear counts, thus only rough appraisals were made in those cases.

Ovaries of two specimens of *F. pygmaeus* were labeled with ^{3}H-uridine. Each frog received an intracoelomic injection of 40 μCi of ^{3}H-uridine (New England Nuclear, specific activity 26.7 Ci/mmole) in amphibian Ringer's solution and was sacrificed 24 hours later. Pieces of ovary were fixed in Bouin's picro-formol or in 3% glutaraldehyde in a phosphate buffer at pH 7.4. The tissues were embedded in Paraplast and cut into sections of 10 μm thickness. After incubation in 10% trichloracetic acid at room temperature for one hour the slides were coated with photographic emulsion (Ilford Nuclear Research emulsion type K-2, in gel form) and stored for 20 days at 4° C. Following development, the slides were stained with Harris' hematoxylin and alcoholic eosin yellow.

RESULTS

Oogenesis in Flectonotus pygmaeus

The ovary of *F. pygmaeus* produces relatively few mature eggs per breeding period (Table I), but each egg accumulates a considerable store of yolk and reaches a final diameter of about 3 mm. The right ovary generally contains more large oocytes than the left (Table I), and sometimes the left ovary appears to be absent altogether. There are, however, individual differences, and in some frogs the left ovary is the larger one.

In the mature ovary there are large, yellowish-white yolky oocytes of 1500 to 3000 μm diameter, a number of previtellogenic oocytes of about 500 μm diameter, and smaller oocytes and oogonia. Following ovulation, oocytes pass through the

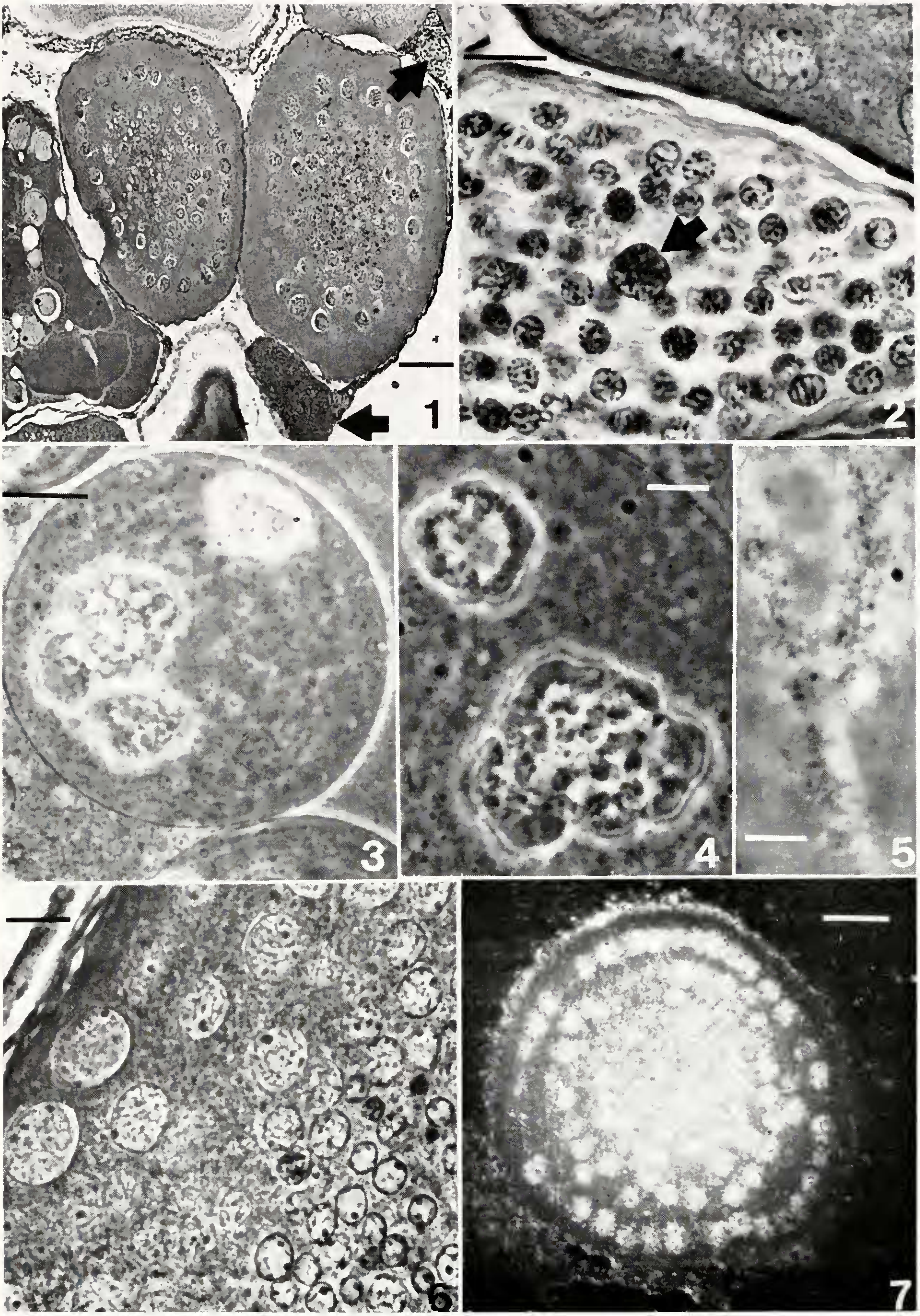

FIGURE 1. Cross section of the ovary of *Flectonotus pygmaeus*. The two centrally located oocytes show nuclei of various sizes, with the larger nuclei distributed towards the periphery and the smaller toward the center of the oocyte. In the oocyte on the left, most nuclei have

oviducts and become covered with a thin coat of jelly similar to that in *Gastrotheca riobambae* (del Pino *et al.*, 1975). During amplexus the eggs are fertilized as they leave the cloaca and are then moved into the pouch; however, the details of the process are not yet known. The oocytes of intermediate size grow rapidly in the mother's ovaries during the period that embryos are being incubated in the pouch and by the time of tadpole birth these oocytes are vitellogenic and about 2 mm in diameter (Table I). Only after birth of the tadpoles do the oocytes attain their mature size of about 3 mm.

The time required for the growth of oocytes from the initial stages to ovulation is not known. Vitellogenesis, however, seems to occur in a matter of a few months, since the oocytes of frogs kept in captivity grew in about five months from 500–800 μm diameter to their mature size. It seems likely that in nature the period of ovarian growth might be shorter.

Oogonial divisions and various stages of oogenesis are easily observed in the adult ovary (Figs. 1, 2). Small oocytes contain from about 1000 to 3000 nuclei (Table II); in larger oocytes the number of nuclei decreases gradually (Figs. 8, 9) until, at later stages, only one nucleus remains (Figs. 10, 11). Early oogenesis occurs within chambers referred to as cysts, following the terminology of King (1908). Oogonial mitoses within cysts appear to be synchronous, but division of the cytoplasm does not always accompany nuclear division; thus occasional multinucleate oogonia are observed. Fusion of the cells of a cyst appears to produce the multinucleate oocyte (Fig. 2). Assuming that all the nuclei in a given cyst are the division products of an original oogonial cell, there are about 11 rounds of mitosis involved in the formation of the multinucleate aggregate that becomes the oocyte. The aggregate measures about 100 μm in diameter and contains some 2000 nuclei (Table II). There are large differences in the number of nuclei from one aggregate to another, but the size of the nuclei is generally uniform; however, there are a few exceptionally large nuclei which may be polyploid (Fig. 2).

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disappeared, and the cytoplasm is not homogeneous. Cysts (arrows) are found frequently. Bar represents 100 μm.

FIGURE 2. Cross section of a cyst in the ovary of *F. pygmaeus*. The size of the nuclei is mostly uniform, but an occasional large nucleus (arrow) is seen. Cell membranes were present between the oogonia of the cyst, but these seem to disappear as the oocyte forms. In the oocyte, nuclei begin to enlarge; two nuclei in the edge of an oocyte can be seen in the upper portion of the figure. The increased size is evident. Bar represents 20 μm.

FIGURE 3. Nucleus from a living preparation of a multinucleate oocyte of *F. pygmaeus*. Note the very large and irregular aggregates of nucleoli. This nucleus had lampbrush chromosomes. In the same oocyte there were other nuclei of smaller size. Bar represents 20 μm.

FIGURE 4. Higher magnification of nucleolar aggregates from a nucleus of a multinucleate oocyte of *F. pygmaeus*. These aggregates are comparable to those of Figure 3. Bar represents 5 μm.

FIGURE 5. Lampbrush chromosome from a living preparation of a nucleus from a multinucleate oocyte of *F. pygmaeus*. Bar represents 5 μm.

FIGURE 6. Cross-section of an oocyte of *F. pygmaeus* with nuclei of various sizes. Large nuclei are located towards the periphery and small nuclei are clustered in the center. Lampbrush chromosomes are found in the larger nuclei. Nucleoli can be seen in most nuclei. Bar represents 20 μm.

FIGURE 7. Autoradiogram showing the incorporation of ^{3}H-uridine into the nuclei of a multinucleate oocyte of *F. pygmaeus*. There was incorporation in both large and small nuclei and in the nuclei of follicle cells. Bar represents 50 μm.

TABLE II

Average number of nuclei in the ovarian oocyte. The number in parentheses indicates the number of oocytes analyzed. In oocytes of 250–500 μm diameter, only the large nuclei were counted (see text).

Diameter of oocyte (μm)	Nuclear diameter (μm)	Number of nuclei/oocyte (± s.d.)	Volume oocyte/volume nuclei (± s.d.)
Flectonotus pygmaeus (nine frogs)			
100– 190 (6)	6.1	2013 ± 961	4.1 ± 1.8
200– 290 (9)	13.9	1484 ± 541	3.9 ± 1.8
300– 390 (4)	21.5	877 ± 95	4.4 ± 0.7*
460 (2)	28.5	818	4.9
950 (1)	180	3	49.0
1200–1300 (3)	393–274**	1	86.3
1500 (5)	390–289	1	114.2
2000–3000 (3)	511–186	1	1186.3
Gastrotheca ovifera (one frog)			
200– 250 (2)	30	194.5	1.9
300– 400 (2)	32	411.5	2.9
410– 500 (5)	40	481.6	3.6
510– 570 (3)	40	390.0	6.7
Gastrotheca sp. (one frog)			
200– 300 (4)	33	71.0	9.1
300– 400 (2)	40	77.0	10.3
500– 550 (3)	33	125.7	32.7
680 (1)	40	107.0	45.9

* The volume of 2000 small nuclei ($650 \times 10^3 \mu^3$) was added to the nuclear volume of each oocyte since small nuclei were not counted.

** Largest and smallest diameters of nuclei of oval shape.

Once meiosis begins, there are no more mitotic divisions within a given cyst. The beginning of meiosis is characterized by moderate enlargement of the oocyte and its nuclei. The nuclear size is generally uniform and the distribution of nuclei seems to be random, except in oocytes with few nuclei; in the latter case most nuclei are located toward the periphery. Inside each nucleus the chromosomes are visible as fine threads, and there are one to a few nucleoli of rounded shape. Elimination of nuclei may begin at this early stage, judging by the presence of numerous small, pycnotic nuclei that stain darkly with hematoxylin; these are found among more normal appearing nuclei located centrally in the oocyte (Figs. 1, 6).

As the oocyte grows, there develop conspicuous differences in nuclear size, correlated with their location in the oocyte. Nuclei located toward the periphery enlarge to a greater extent than those located more centrally. These differences can be detected in oocytes of 250 μm diameter and are soon obvious. In oocytes of 300 μm the largest nuclei, located just beneath the cortex, measure about 20 μm;

nuclei located somewhat deeper are smaller; those located in the center are the smallest (6 to 7 μm) (Figs. 1, 6). Large and medium size nuclei contain chromosomes in the lampbrush state (Fig. 5); in smaller nuclei the chromosomes are visible as fine threads but other characteristics could not be determined. The nucleoli of each nucleus seem to associate into a few large masses of irregular shape, nucleolar aggregates (Figs. 3, 4), which are common to all nuclei, except possibly those that are pycnotic. Growth of the oocyte is accompanied by further enlargement of the peripheral nuclei (Table II) and a decrease in their number. Correlated with the smaller number of large nuclei there seems to be an increase in the number of medium size nuclei and small nuclei. The latter nuclei are very abundant in oocytes of 300 to 700 μm diameter and are clustered in the center of the oocyte (Fig. 6); they are so numerous and closely packed that they are impossible to count, but their number seems to vary. Owing to the difficulty of counting the centrally located nuclei, only the larger nuclei were counted in oocytes of 250 to 500 μm diameter (Table II). The decrease in total number of nuclei during this period is thus more gradual than is suggested by the figures in Table II, which shows exclusively the decrease in the number of larger nuclei. Indeed, the number of nuclei appears to remain rather constant from the formation of the oocyte up to a size of about 400 μm and possibly later. In one oocyte of 390 μm diameter, for example, the number of small nuclei was estimated to be about 2500 with a mean diameter of 8.6 μm; in the same oocyte there were about 750 large nuclei with diameters of about 25 μm.

In oocytes of 700 to 800 μm the chromosomes are no longer in the lampbrush condition and cannot be seen in most nuclei. All nuclei, including the smallest ones, contain large and conspicuous nucleolar aggregates of irregular shape. In oocytes of 800 to 900 μm, the number of large and small nuclei decreases markedly (Figs. 8, 9); during this process the large nuclei decrease somewhat in volume and become pycnotic before finally disappearing. Associated with nuclear disappearance there are changes in the appearance of the cytoplasm, presumably the result of the addition of nuclear material (Figs. 1, 8, 9). This modified cytoplasm is originally distributed unevenly in the oocyte, but the several areas of differing appearance eventually join and give a more homogeneous character to the cytoplasm. Numerous vesicles are formed slightly later and the oocyte eventually becomes almost filled with them. When only 1 to 10 large nuclei remain in the oocyte, yolk platelets begin to appear. At this time the diameter of the oocyte is approximately 1000 μm.

The remaining nuclei enlarge greatly and reach a diameter of about 180 μm. These large nuclei occupy the central region of the oocyte, in contrast to the peripheral location of large nuclei during earlier stages. After oocytes reach about 2 mm in diameter, only one nucleus is found. This germinal vesicle is originally spherical and is located near the center of the cell (Fig. 10); as vitellogenesis proceeds, it moves toward the periphery of the oocyte and becomes ovoid (Fig. 11). It is considerably larger than any of the preceding nuclei.

In the last of the multiple nuclei and in the germinal vesicle there are sometimes accumulations of basophilic material just inside the nuclear envelope (Fig. 11); the nature of this material is unknown. In each of the final nuclei, the nucleolar aggregates become very large before disappearing (Fig. 10); these seem to be re-

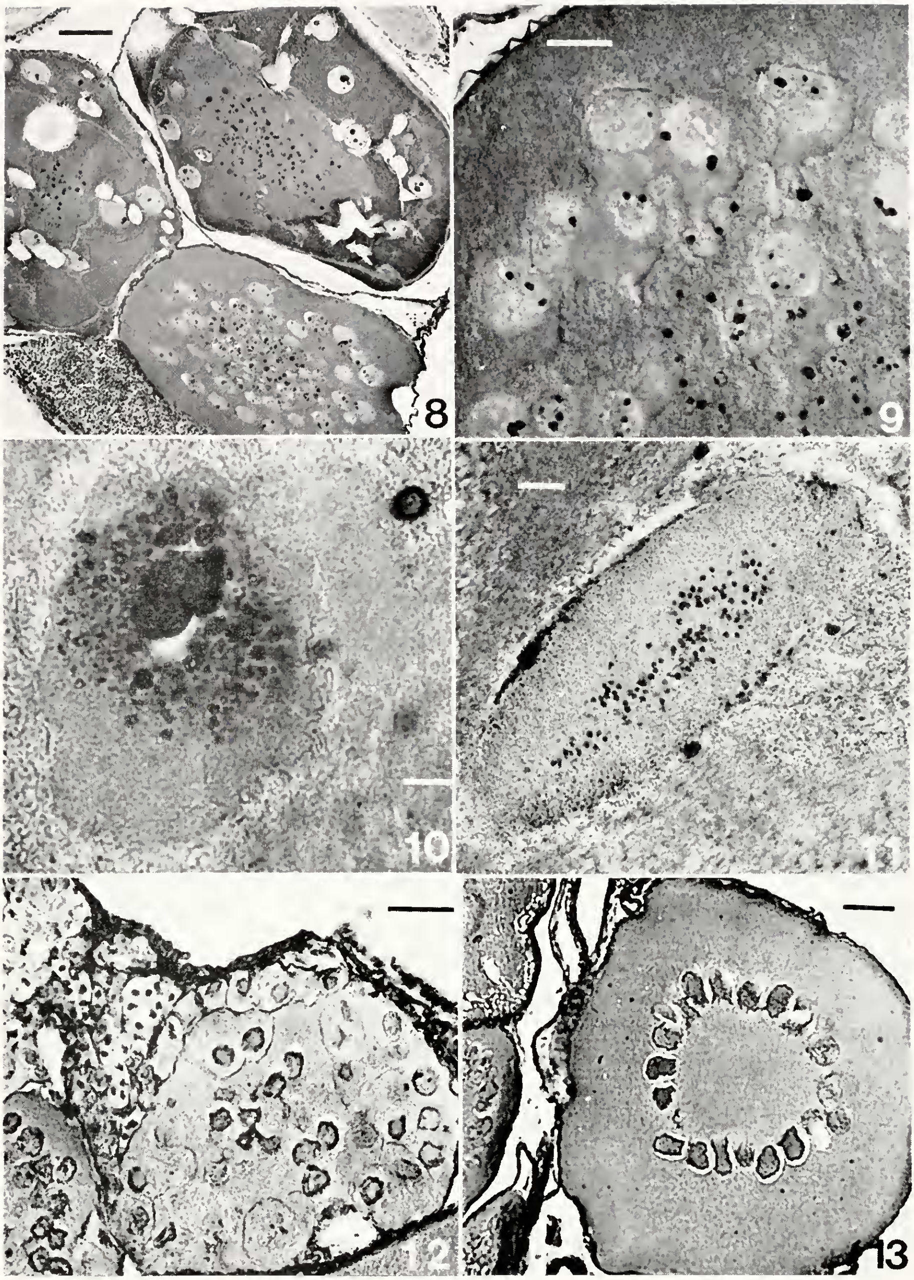

FIGURE 8. Cross section of ovary of *F. pygmaeus*. In the two upper oocytes there is great reduction in the number of nuclei. Large nuclei shrink and become pycnotic before disappearing. Both oocytes show also the uneven distribution of cytoplasm modified by nuclei. In the lower portion of the figure there is a multinucleate oocyte with nuclei of various sizes and a follicle of atresia. Bar represents 100 μm.

placed gradually by small ovoid corpuscles that might be nucleoli. In addition, there are many spherical corpuscles, much smaller than the usual nucleoli (Fig. 11). Both types of corpuscles are present in the germinal vesicle of the largest oocytes. The chromosomes of this stage were not found.

The yolk-filled cytoplasm of large ovarian oocytes and ovulated eggs contains numerous transparent round vesicles that are distributed almost uniformly. The animal pole of the egg is almost devoid of both vesicles and yolk granules; it is within this zone that the germinal vesicle is located. After ovulation, the germinal vesicle could not be found in the only such egg that was available, suggesting that ovulation and germinal vesicle breakdown occur at about the same time.

Small nuclei of large oocytes gave a strong Feulgen reaction, while lampbrush chromosomes, nucleoli and nucleolar aggregates of larger nuclei reacted weakly. Although nucleolar aggregates did not ordinarily give a positive reaction, there was sometimes a positive reaction from large bodies that seemed to be nucleolar aggregates. In the germinal vesicle, only the ovoid corpuscles reacted with Feulgen, and these only faintly.

All nuclei in oocytes up to about 500 μm in diameter incorporated ^{3}H-uridine (Fig. 7). Uridine incorporation was detected in association with chromosomes during the early stages of meiosis; later, however, uridine seemed to be incorporated almost exclusively by the nucleoli. In large oocytes, where the nuclear number was greatly decreased, there was slight or no incorporation of label.

Nuclear changes during oogenesis in Gastrotheca ovifera *and other marsupial frogs*

During oogenesis the oocyte becomes multinucleate in *G. ovifera* and other species of marsupial frogs (Table III), but the number of nuclei per oocyte in these frogs is smaller than that in *F. pygmaeus* (Table II). In *G. ovifera* the nuclear number reaches about 500, presumably the result of approximately nine mitotic divisions of the original oogonium; in *Gastrotheca* sp. (Venezuela) there are only about 100 nuclei, corresponding to fewer than seven divisions. The number of nuclei in oocytes of other species with multinucleate oocytes (Table III) was not counted, but it seems comparable to that of *G. ovifera* or *Gastrotheca* sp., with the exception of *Hemiphractus johnsoni*, which has fewer nuclei. There was a maximum of four nuclei per oocyte in the only ovary of this species that was examined.

FIGURE 9. Detail of the changes in large nuclei prior to disappearance. The cytoplasm adjacent to nuclei becomes modified and the nuclei shrink before becoming pycnotic. Bar represents 50 μm.

FIGURE 10. Cross section of the nucleus from a mononucleate oocyte of *F. pygmaeus*. This nucleus has enlarged greatly and shows very large aggregates that will be replaced later by smaller corpuscles. Bar represents 25 μm.

FIGURE 11. Cross section of the single germinal vesicle of an oocyte of *F. pygmaeus*. There are ovoid corpuscles towards the center and very abundant spherical entities of smaller size. The chromosomes could not be seen. Note the basophilic accumulations of material just inside the nuclear envelope. Bar represents 25 μm.

FIGURE 12. Cross section of a cyst in the ovary of *Gastrotheca ovifera*. Most oogonia are mononucleate; these will fuse to give a multinucleate oocyte with about 500 nuclei. Bar represents 50 μm.

FIGURE 13. Cross section of an oocyte of *G. ovifera*. The enlarged nuclei are distributed in a layer. Nuclei are absent from the central region. Bar represents 100 μm.

TABLE III

Type of oogenesis and development at birth in marsupial frogs.

Species	Egg diameter (mm)	Development	
		Tadpole	Froglet
Mononucleate type of oogenesis			
Cryptobatrachus			
C. fuhrmanni			+
Flectonotus			
F. fissilis	3	+	
Gastrotheca			
G. argenteovirens			
G. cavia	3	+	
G. christiani	4		+
G. excubitor	6		+
G. gracilis	3	+	
G. lojana			
G. marsupiata	2.5	+	
G. mertensi	5		+ (1)
G. monticola		+	
G. ochoai	5		+
G. peruana	3	+	
G. plumbea	4		+ (1)
G. riobambae	3	+	
G. testudinea	3.5		+
G. sp. (LSUMZ-32049)			+
G. sp. (FMNH-39889)	4		+
Hemiphractus			
H. bubalus			+ ?
H. fasciatus	7		+
H. proboscideus			+
H. scutatus	10 (2)		+
Multinucleate type of oogenesis			
Amphignathodon			
A. guentheri			+
Flectonotus			
F. pygmaeus	3	+ (3)	
Gastrotheca			
G. ceratophrys	8		+
G. cornuta			+
G. griswoldi	8		+
G. microdisca			+
G. ovifera	8		+
G. weinlandii			+
G. sp. (Venezuela)	7		+
Hemiphractus			
H. johnsoni			+
Stefania			
S. scalae	9		+
Total		12	21

Number of species: 33. (1) Young are born as froglets or towards the end of metamorphosis. (2) Egg diameter from Trueb, 1974. (3) Born as advanced tadpole.

In *G. ovifera,* oogonial divisions within a cyst are apparently synchronous, as in *F. pygmaeus,* and most oogonia contain a single nucleus (Fig. 12). Oogonia with 2 to 4 nuclei are rare, in contrast to *F. pygmaeus,* where many oogonia seem to be multinucleate. In *G. ovifera* the oocyte seems to be formed by fusion of mononucleate oogonia of a cyst. With the onset of meiosis, nuclei enlarge, form lampbrush chromosomes and later, conspicuous nucleolar aggregates. Most nuclei, and possibly all of them, become arranged toward the peripheral region of the oocyte, forming one or two layers of nuclei (Fig. 13). The nuclei of the internal layer are slightly smaller than those toward the periphery. In addition, there are a few pycnotic nuclei of small size that are found just internal to the larger nuclei. Pycnotic nuclei have also been observed in the periphery and sometimes in the central region of the oocyte. In most cases, however, there are no nuclei in the central part.

As the oocyte grows, there is a gradual disappearance of nuclei and, toward the beginning of vitellogenesis, there is apparently a period of more rapid nuclear disappearance. The onset of vitellogenesis is accompanied by changes in the cytoplasm of the oocyte that are comparable to the changes described for *F. pygmaeus. Gastrotheca ovifera* produces very large oocytes (approximately 8 mm diameter) and it seems that the oocyte contains multiple nuclei until a large size is attained: oocytes of about 2 mm in diameter do not have yolk platelets and still have numerous large nuclei.

Eleven of the 33 species of marsupial frogs examined (Table III) are characterized by the presence of multiple nuclei in the oocyte during the early stages of oogenesis. Although the formation of the oocyte in some of these species was not observed, those that were studied show similarities to *F. pygmaeus* or *G. ovifera.* In the majority, the nuclei become arranged toward the peripheral region of the oocyte and enlarge. The central region does not have nuclei in most instances, but occasionally a few small pycnotic nuclei can be seen. Each nucleus contains lampbrush chromosomes and nucleolar aggregates. The number of nuclei decreases gradually as the oocyte grows; the multinucleate condition, however, is prolonged into the vitellogenic period, as in *G. ovifera.*

Discussion

There is little doubt that the multiple entities described here as nuclear are either nuclei or derivatives of nuclei. The presence of visible chromosomes in many of the nuclei and the incorporation of labeled uridine attest to this view, as do ultrastructural studies (in preparation) which reveal the presence of a typical nuclear envelope. What is unclear is whether the nuclei all contain chromosomes and whether the number of chromosomes in each is the same. The extreme variation in size of the nuclei suggests that the chromosome number may not be the same, or that the condition of the chromosomes may vary from the large nuclei to the small. Although no information is available as to the DNA content of these multinucleate oocytes or the DNA content of individual nuclei, the situation suggests a massive overall amplifiation of the oocytes' DNA, considerably greater than that occurring in the eight-nucleate oocytes found in *Ascaphus truei* (Macgregor and Kezer, 1970). All nuclei are presumably engaged in synthesizing RNA, at least prior to the final stages where only a single germinal vesicle or a few large nuclei are present. It appears, then, that these multinucleate oocytes represent a remarkable case of a sort of endopolyploidy of high degree, even if the nuclei contain abnormal chromosome complements.

We have no explanation to propose for the widespread occurrence of multinucleate oocytes in marsupial frogs; moreover, we have been unable to find a clear relationship between the occurrence of the multinucleate condition in these species and such characteristics as the final size of the egg or the pattern of reproduction. However, there does seem to be some correlation between the multinucleate condition, production of unusually large eggs, and development of young to the froglet stage in the maternal pouch. A notable exception is *F. pygmaeus,* in which the eggs do not seem unusually large and in which the young are kept in the pouch only until they are advanced tadpoles. An egg of 3 mm, however, might be considered large for such a small frog (30 mm snout-vent length), since other frogs with multinucleate oogenesis measure from 40 to 80 mm (snout-vent length). Some frogs with mononucleate oogenesis, however, are of similar size and produce eggs that are as large as those of species with multinucleate oocytes. In both the multinucleate and mononucleate groups there are species that give birth to tadpoles and species that give birth to froglets (Table III). One generalization that may be important is that the marsupial frogs, regardless of nuclear number during oogenesis, produce eggs that are very large by usual amphibian standards, but very few in number. Furthermore, reproduction seems to be exceptionally efficient: almost all eggs produced appear to be fertilized and undergo development. In *F. pygmaeus,* for example, the number of large eggs in the ovary is small, and corresponds closely to the number of embryos in the pouch.

The total nuclear volume increases considerably during early oogenesis, but the concomitant great increase in oocyte volume results in what seems to be a rather constant ratio between the two during the previtellogenic period (Table II). No firm conclusions can be reached, however, since the estimations of volume are subject to large error and since there is also great variability in the number of nuclei per oocyte. During the vitellogenic phase, oocyte growth is not paralleled by an increase in nuclear volume; thus, the ratio of oocyte to nuclear volume increases greatly toward the end of oogenesis (Table II). Little information is available regarding ratios between oocyte volume and nuclear volume in the Amphibia, but in the mononucleate oocytes of *Rana pipiens* (Parmenter *et al.,* 1960) the ratio seems to increase earlier than in *F. pygmaeus.* In any case, however, the multinucleate condition obviously results in an enormous nuclear surface area, particularly in oocytes of *F. pygmaeus,* with their large number of centrally located nuclear entities.

The multinucleate condition in the oocytes described in this report differs in several notable ways from that in *Ascaphus truei* (Macgregor and Kezer, 1970). The maximum number of nuclei in *Ascaphus* oocytes is usually eight, although many ovaries contain a few oocytes with sixteen nuclei (Kezer, personal communication); the condition arises from failure of cytokinesis to occur following nuclear division. In *F. pygmaeus, G. ovifera,* and *Gastrotheca* sp., however, the number of nuclei reaches the hundreds, and the condition seems to originate through the disappearance of cell membranes between adjacent cells within a cyst. Another difference is the fact that in *Ascaphus* the multinucleate condition persists until late oogenesis, while in at least some of these marsupial frogs the multinucleate condition lasts only until about the time yolk formation begins. In both situations, however, the multiple nuclei contain chromosomes in the lampbrush condition, and there are similar nucleolar features. In neither situation is there good evidence as to the mechanism by which the number of nuclei is finally reduced to one.

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Summary

The occurrence of multinucleate stages during oogenesis appears to be widespread in the marsupial frogs of South America. In some species the number of nuclei or nucleus-like entities per oocyte is estimated to be as high as 2000, but the number in other species may be considerably lower; some species have only a single oocyte nucleus. In all cases it seems that only a single nucleus remains as the oocyte approaches maturity. The situation suggests a massive general amplification of the genome of the multinucleate oocytes that is much greater than has yet been reported. Possible relationships between the occurrence of the multinucleate condition and such features as egg size and reproductive pattern are discussed, but no final conclusions can be made on the basis of the evidence presently available.

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